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Large-scale quantitative isolation of pure protein N-linked glycans

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ABSTRACT

Glycoproteins are biologically active proteins of which the attached glycans contribute to their biological functionality. Limited data is available on the functional properties of these N-glycans in isolation, without the protein core. Glycan release, typically performed with the PNGase F enzyme, is achieved on denatured proteins in the presence of detergents which are notoriously difficult to be completely removed. In this work we compared two methods aiming at recovering N-glycans in a high yield and at high purity from a PNGase F glycoprotein digest of bovine lactoferrin. Detergents were removed from the digest by two separate approaches. In the first approach, protein and glycans were precipitated with acetone and the detergent containing supernatant was discarded. In the second approach, detergent was removed by adsorption onto a polystyrene resin. Following detergent removal, the glycans were further purified by a sequence of solid phase extraction (SPE) steps. Both approaches for detergent removal yielded a final glycan purity above 85%. Recovery of the glycans from lactoferrin was, however, much lower when utilizing acetone precipitation versus the polystyrene resin; 52% versus 85% respectively. A more detailed analysis of the acetone precipitation step revealed a loss of shorter oligomannose structures specifically. A loss of glycans of lesser complexity (oligomannose and biantennary structures) was also observed for other glycoproteins (RNase B, porcine thyroglobulin, human lactoferrin). These results indicate that acetone precipitation, a commonly used step for small-scale glycan purification, is not suitable for all target glycoproteins. The polystyrene resin detergent removal step conserved the full N-glycan profile and could be applied to all mammalian glycoproteins tested. Using this optimized protocol, large-scale quantitative isolation of N-glycan structures was achieved with sufficient purity for functional studies.

1. Introduction

Protein glycosylation is a co- and posttranslational modification of protein structures with carbohydrate moieties. These glycans are commonly divided into N-linked and O-linked glycans based on their location. O-linked glycans, bound to a serine or threonine residue, differ greatly in structure when compared to N-linked glycans, bound to an asparagine residue [1].

Glycans are responsible for many functional properties of glycoproteins, including a) cell adhesion, b) protein folding, c) protection against degradation, d) solubility and e) immune modulatory effects [2,3]. Determining the functionality of glycoproteins and their glycan structures usually involves various approaches. For example their interactions with lectins or glycan binding proteins can be studied [4,5]. Modification of their glycan profile can also be performed by intervention in the glycosylation pathway, either by genetic engineering [6] or pharmacologically [7]. In addition, glycans can be modified by glycosidase enzyme treatments. This latter approach has been applied on glycoproteins which, after modification, have been used for both *in vivo* [8] and *in vitro* studies [9]. In other cases genetic mutants are made of the glycoprotein to generate a modified glycan profile [10], or even a non-glycosylated variant [11,12]. Chemical modification of isolated glycoproteins can also be performed [13], followed by functionality testing.

Non-glycosylated variants of glycoproteins thus can be obtained and used for functional analysis of the protein moieties on their own. Glycoprotein functionality can also be studied following modification of the glycan pattern, or by comparing glycoprotein variants from different sources. However, structure-function relation information on isolated N-glycans is currently very limited. Other carbohydrate structures, such as free oligosaccharides in human milk (*h*MOS), have many proven functions [14]. hMOS and galacto-oligosaccharides (GOS) have been shown to possess prebiotic [15] and Toll-like receptor (TLR) stimulating activities [16]. Recently, N-glycan structures released from

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bovine colostrum whey were described to be selectively consumed by bifidobacteria [17]. Free glycans potentially have very different functional properties than in glycoconjugate form and are therefore an interesting target for further study.

The limited number of studies that investigate the function of isolated N-glycans is at least partly due to the difficulty of isolating N-glycans in sufficient quantity and purity. Glycoprofiling studies require ng to μg quantities of N-glycans, which can be released from μg quantities of protein. Structure-function analyses, however, will usually require several mg of purified N-glycan products. The N-glycan portion of glycoproteins ranges typically from 2 to 20% by weight [18,19]. Clearly, to isolate mg quantities of N-glycans, glycoproteins have to be available on a 100 mg to gram scale. Many glycoproteins are low in abundance and obtaining enough pure protein to generate significant quantities of N-glycans can be costly and time consuming. Also when the glycoprotein of interest is readily available, the methods available for small-scale isolation of N-glycans have to be adapted to accommodate the larger scale digests.

The first step in the isolation of N-glycans is their release from the glycoproteins, either chemically or enzymatically. Methods for chemical release of glycan structures use harsh and toxic chemicals. In addition, undesirable glycan modifications may take place, such as the loss of *N*-acetyl and *N*-glycolyl groups [20]. Enzymatic release of N-glycans from denatured proteins is performed under mild conditions, without any damage to the glycan structures, and is therefore preferred. Enzymatic cleavage protocols still require denaturing agents and detergents that may influence any subsequent functional biological study. Therefore, isolation of N-glycans samples also requires proper purification protocols.

Purification of glycans can be performed either with native glycans, or after derivatization with a functional group that facilitates detection and purification. Many options exist for the purification of derivatized N-glycans, including capture on solid phase materials such as cellulose, cotton, and ZIC-HILIC materials [21,22]. Additional options for labeled N-glycan purification include sequential HPLC steps [23] and capturing on PVDF membranes [24].

Purifying native glycans is much more difficult due to the inherent low retention on reversed phase and HILIC materials. Capture on graphitized carbon material is most commonly used [25], or a precipitation step is performed with acetone [26]. Detergents commonly used in glycoprotein digests (SDS and NP-40), are notoriously difficult to be fully removed. Dialysis or ion exchange are often not compatible with the smaller size of the glycans (dialysis) or the nature of the detergent (non-ionic versus ionic) used. Detergents can also be removed by adsorption onto polystyrene beads, such as the commercially available Bio-Beads SM2 (Bio-Rad) or Amberlite XAD-2 (Sigma) [27,28].

While individual purification methods are often sufficient to yield a glycan sample clean enough for profile analysis, some residual contamination with protein or detergent is often present. In order to yield a completely pure sample for functional analysis, individual purification methods will have to be combined.

This paper describes the development of a large-scale quantitative isolation method for recovering N-glycans from a glycoprotein digest. Two approaches for removing detergents from N-glycans are compared in detail. Acetone precipitation, described in earlier literature as suitable for N-glycan precipitation [26], is investigated in more detail to evaluate its use with various glycoprotein digests.

2. Results

2.1. Large-scale digestion and purification of N-glycans

PNGase F digests of 450 mg (approach A) and 1000 mg (approach B) glycoprotein were prepared and subjected to different methods of N-glycan purification. The main difference was the method chosen to remove the detergent from the samples. Detergent was removed either by acetone precipitation (approach A), or by adsorption onto Bio-Beads SM-2 (approach B). In case of acetone precipitation, proteins and glycans are precipitated together, acetone is discarded and the glycans are extracted from the pellet with 60% methanol [26]. This step removes a large part of the protein and the methanol extract was therefore directly used for the subsequent steps. In case of Bio-Beads SM-2 detergent removal, the detergent was adsorbed onto the beads, leaving protein, glycans and buffer salt in solution. The proteins in the solution were removed by an additional filtration step to avoid overloading the SPE columns in the subsequent cleanup.

Following detergent removal, the central part of both approaches was a sequence of reversed phase (C18) and graphitized carbon SPE steps. The combination of C18 and graphitized carbon SPE was performed with 5 g packed columns in sequence (approach A), or extractions on individual prepacked cartridges (approach B).

In order to be able to determine the yield and purity of the obtained glycans, a theoretical yield was calculated first. Our target protein for isolation of N-glycans was bovine lactoferrin, a glycoprotein with a carbohydrate content of approximately 6.7–11.2% consisting solely of N-glycans [29,30]. The carbohydrate content of the bovine lactoferrin used in this study was determined by monosaccharide analysis and was found to be 7.7 ± 0.4%, which is consistent with these earlier reports. Using this 7.7% value, the expected amount of pure carbohydrate was calculated and compared with the final purified product obtained.

After the final graphitized carbon purification step, an N-glycan purity of approximately 20% and 40% was calculated for approach A and B, respectively (data not shown). A final wash of the lyophilized product with 100% acetone resulted in a purity of 89–103% (carbohydrate per weight) (Table 1). To verify that N-glycans were not lost in the 100% acetone wash step, this wash fraction was dried under N_2 and subjected to monosaccharide analysis. Glycan loss during this step was negligible, determined at < 1% of the total N-glycan product weight (data not shown).

NMR spectroscopy analysis of the final product revealed that carbohydrate structural reporter groups were present and that the product was completely free of protein and detergent (Fig. 1, Table 2, Scheme 1). No other structures could be identified by one-dimensional ^{1}H NMR analysis. The remaining 0–11% therefore consists of compounds that

Table 1
Comparison of the yield, purity and recovery of N-glycans obtained from bovine lactoferrin with the protocol including acetone precipitation (approach A), or Bio-Beads SM-2 (approach B).

	Initial protein (mg)	Yield final product (mg)	Purity (glycan/weight)	mg recovered	Recovery
Acetone protocol	450	20.0	89% ± 3.9 (duplicate)	18.0	52%
Maltoheptaose (control)	N.A.	N.A.	79%	N.A.	N.A.
Bio-Beads SM-2 protocol	1000	65.4	103% ± 1.6 (triplicate)	65.4	85%
Maltohexaose (control)	N.A.	NA	98% ± 0.2	N.A.	N.A.

N.A. = Not applicable.
Purity and recovery were determined by duplicate analysis of the lyophilized products. Recovery was estimated based on the average N-glycan weight percentage of lactoferrin (7.7%). Recovery was calculated using the weights of the final products, corrected for purity. A standard oligosaccharide (maltoheptaose or maltohexaose, ≥90% purity) was chosen as a performance control of the monosaccharide analysis.

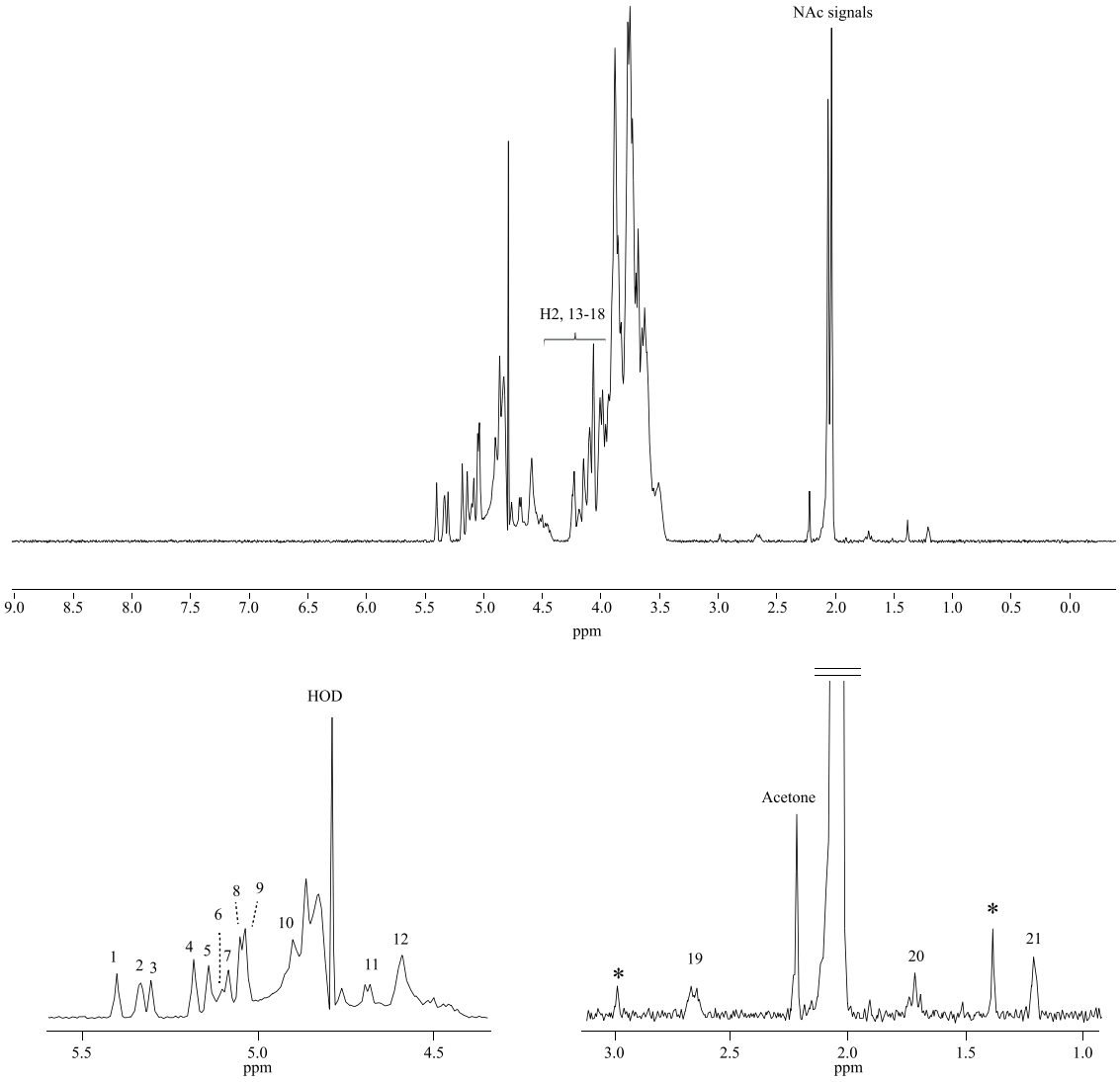


Fig. 1. One dimensional 1H NMR spectra of the purified N-glycan profile of bovine lactoferrin. The full spectrum is shown at the top half, with relevant sections magnified in the lower half. Known structural reporter groups are annotated in the spectra and described in Table 2. Signals corresponding to unknown compounds are annotated with *.

were not detected by NMR spectroscopy, and may include salts, or any water that remains after lyophilization, or is easily attracted due to the high hygroscopic nature of carbohydrate structures [31]. An underestimation also may have occurred during the monosaccharide analysis, since a similar purity was obtained when analyzing the standard (maltohexaose or maltoheptaose) sample (98 and 79%, respectively; Table 1).

2.2. Recovery and purity of the obtained N-glycans from both approaches

The recovery and purity of N-glycans after the procedure including acetone precipitation (approach A) or Bio-Beads SM-2 protocol (approach B) were compared (Table 1). While the purity of the glycans was comparable, the recovery of the Bio-Beads SM-2 protocol was significantly higher.

With both methods no significant glycan losses were observed in the C18 and graphitized carbon steps, neither with bulk columns nor when using prepacked cartridges (data not shown). Since the supernatant, containing 80% acetone and detergent, is discarded during the acetone precipitation protocol, we speculated that the loss of N-glycans occurred in this step.

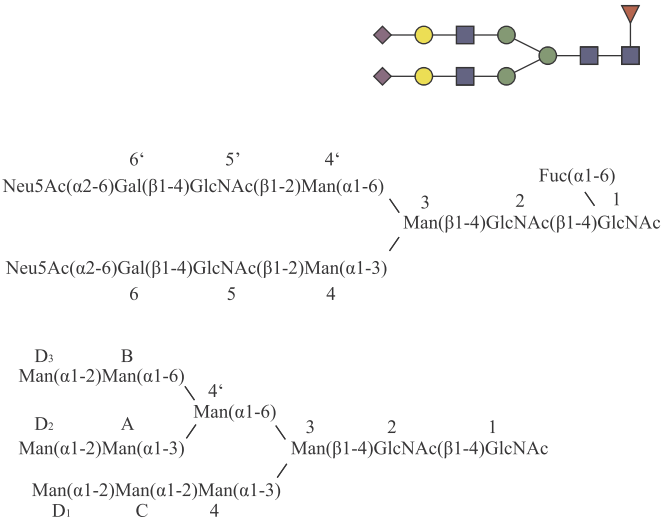
2.3. Glycoprofiles of various glycoproteins after acetone precipitation

The protein used for the large-scale isolations, bovine lactoferrin, is a glycoprotein with a limited spectrum of N-glycans. The main constituents are glycans of the oligomannose type, $Man_5GlcNAc_2$ (Man-5) to $Man_9GlcNAc_2$ (Man-9), with minor levels of hybrid and complex type of structures [29]. Analysis of the glycoprofiles of pellet and acetone fractions confirmed the presence of lactoferrin glycan structures in the acetone fractions (Fig. 2A, red line), which are normally discarded. The oligomannose structures (Man-5 to Man-9) were readily identified in the glycoprofile of lactoferrin based on the fixed increase in retention time with each mannose added to the glycan chain. When the profiles of the pellet and acetone fractions were overlaid, differences became clearly apparent. More than 50% of the total amount of Man-5 remained in the acetone fraction, while larger structures such as Man-8 and Man-9 were precipitated more efficiently, with only limited amounts observed in the acetone fraction. The precipitation efficiency thus directly correlated with the length and complexity of the glycan structures (Fig. 2A).

Bovine lactoferrin has a limited spectrum of N-glycans, and the observed loss of glycans in the acetone precipitation step appeared to be

Table 2
Structural reporter signals found in the 1D ^{1}H NMR spectra of the purified N-glycan profile of bovine lactoferrin.

	ppm	Annotation
1	5.40	A (Man-9)
2	5.34	4 (Man-9)
3	5.30	C (Man-9)
4	5.18	1a (GlcNAc 1)
5	5.14	B (Man-9)
6	5.10	4/A (Man-5/6/7/8)
7	5.08	A (Man-8)
8	5.05	C (Man-6)
9	5.04	D1 (Man-7"/Man-8)
10	4.90	B/4' (Man)
11	4.68	1b (GlcNAc)
12	4.59	2 (GlcNAc)
13	4.23	H2 (Man)
14	4.15	H2 (Man-4′)
15	4.10	H2 A + C (Man)
16	4.06	H2 D1-D3 (Man)
17	4.01	H2 B (Man)
18	3.99	H2 B
19	2.67	Neu5Ac
20	1.72	Neu5Ac
21	1.21	Fuc



Scheme 1. Schematic representation of an oligomannose Man-9 structure (bottom), and a biantennary structure (top), with their residue coding. Additionally, a symbolic representation of the binantennary structure is given.

limited to glycans of low complexity. The efficiency of the acetone precipitation step was investigated in more detail, using a selection of proteins encompassing a full range of glycan-types, up to tetra-antennary structures. The glycoprofiles of their pellet and acetone fractions were analyzed and compared.

The incomplete precipitation of the oligomannose type glycans was also seen with RNase B (Fig. 2B), a well characterized glycoprotein which carries almost exclusively oligo-mannose (Man-5 to Man-9) type glycans [32,33]. Comparing the profiles obtained with RNase B, a loss of more than 50% of the total amount of the Man-5 glycan was observed in the acetone fraction, while the Man-9 glycan was predominantly recovered from the pellet fraction (Fig. 2B).

With ovalbumin, glycan structures smaller than the Man-5 glycan were also observed in the acetone fraction. While these structures typically do not originate from ovalbumin itself, but from co-isolated proteins [34], they give useful information for the evaluation of the acetone precipitation step. Analysis of the glycoprofiles of ovalbumin demonstrated that these smaller structures precipitated even more poorly, with close to 100% remaining in the acetone fraction (Fig. 2C).

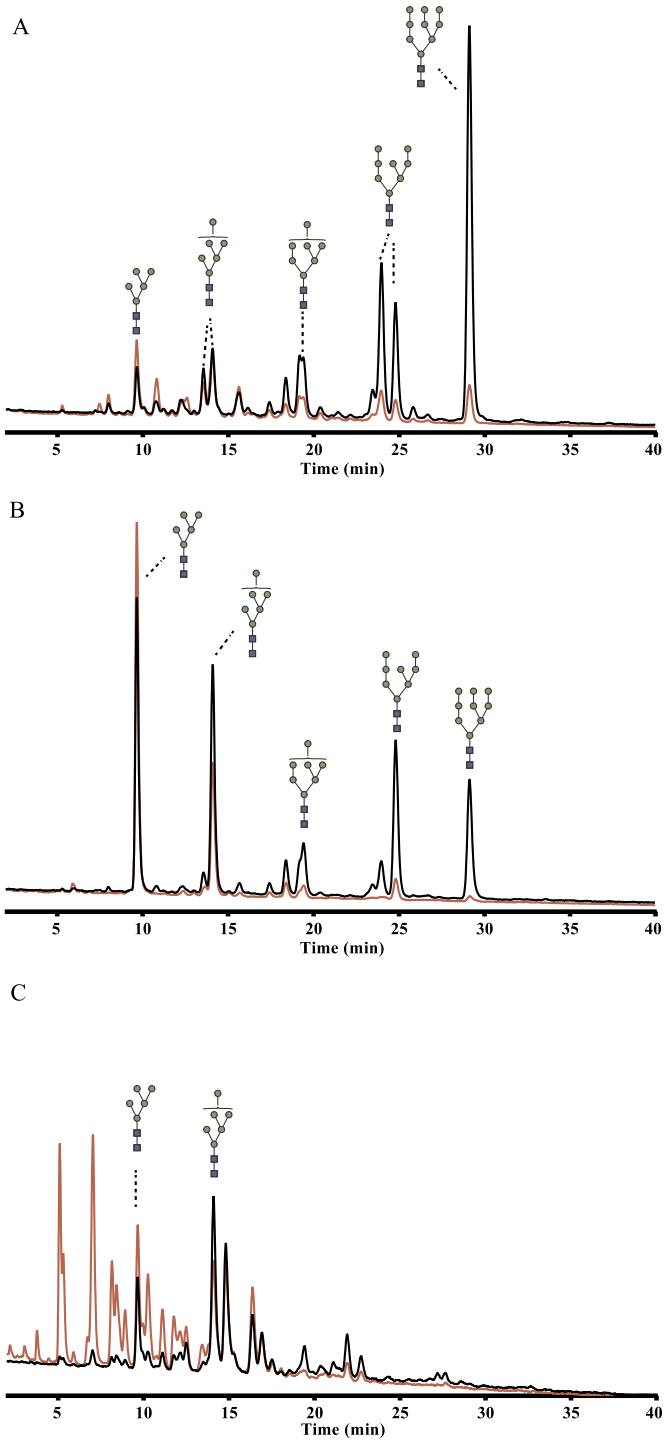


Fig. 2. Glycoprofiles obtained in the acetone (red line) and pellet (black line) fractions of Bovine lactoferrin (A), RNase B (B) and Ovalbumin (C). Oligomannose type glycans are annotated. Note that during normal acetone precipitation processing, the acetone fraction is discarded. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The observed pattern of incomplete precipitation was also seen upon analysis of the glycoprofiles of the acetone and pellet fractions of thyroglobulin and human lactoferrin (Fig. 3). Thyroglobulin is a glycoprotein expressing glycans of the oligomannose-type, as well as sialylated di- and triantennary structures with and without core fucosylation [35,36]. The latter structures are also present on human lactoferrin [37]. With both thyroglobulin and human lactoferrin,

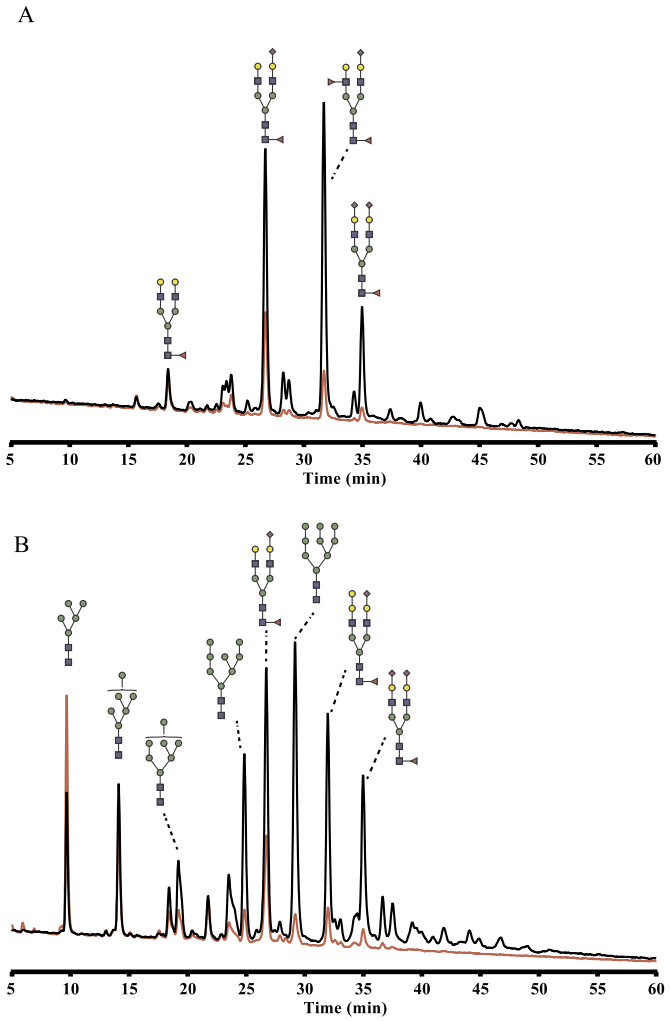


Fig. 3. Glycoprofiles obtained in the acetone (red line) and pellet (black line) fractions of human lactoferrin (A) and porcine thyroglobulin (B). Annotated structures were confirmed by exoglycosidase assays (supplemental information). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

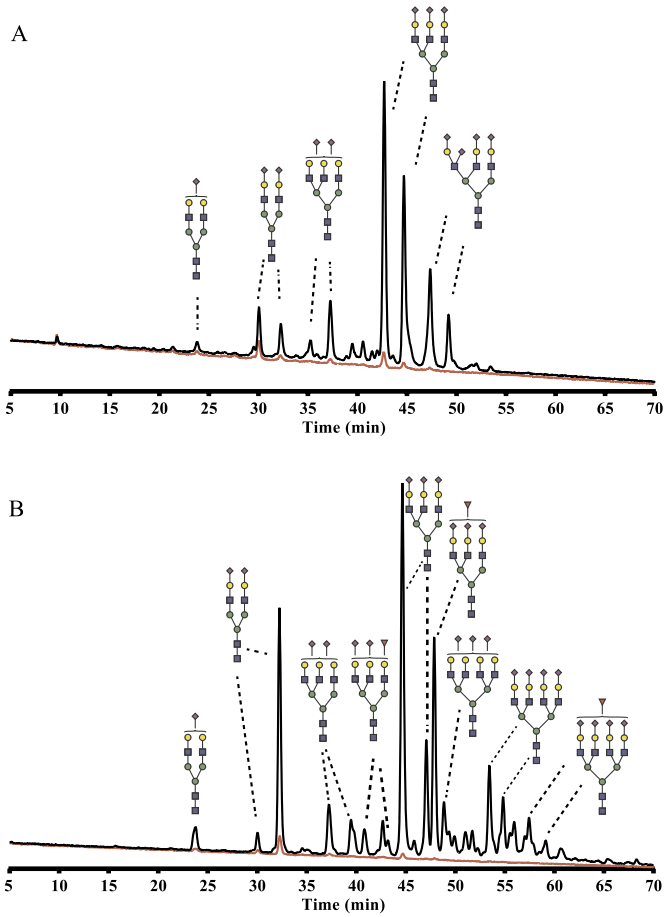


Fig. 4. Glycoprofiles obtained in the acetone (red line) and pellet (black line) fractions of fetuin (A) and α-acid glycoprotein (B). Structures were annotated using the publications of [45,51,52], supported by an exoglycosidase assay for AGP (supplemental information). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

precipitation efficiency was not solely dependent on the glycan size. For example, Man-8 and Man-9, consisting of 10 and 11 monosaccharides respectively, precipitated more efficiently than NeuAc(GalGlcNAc)$_2$Man$_3$(GlcNAc)$_2$Fuc (FA2G2S1) and NeuAc(GalGlcNAc)2Man3(GlcNAc)$_2$Fuc (FA2G2S2) (11 and 12 monosaccharides, respectively).

Calf serum fetuin and human α-acid glycoprotein (AGP) are glycoproteins known for their complex di, tri and tetraantennary structures with high sialylation levels [19,38–41]. Analysis of their glycan profiles confirmed that the more complex glycan structures precipitated best resulting in their highest recovery (Fig. 4). With the exception of the smaller biantennary structures, the larger complex type glycans almost fully precipitated and were recovered in the pellet fraction (Fig. 4, black line).

2.4. *Effect of incubation conditions on the precipitation of Man-5 and Man-9*

In the glycoprofiling experiments and for the large-scale isolation of N-glycans from lactoferrin, we used detergent NP-40 in a phosphate buffer at pH 5.5 for glycan precipitation, conditions which differ from those described by Verostek *et al.* To investigate the effects of these protocol modifications on precipitation efficiency, an additional precipitation experiment was performed. Instead of deglycosylating the glycoproteins, 100 ng of Man-5 and Man-9 were added to the incubations in order to be able to quantify the distribution of these glycans in the pellet and acetone fractions. Precipitations were performed in phosphate buffer with denaturing agents, adjusted to pH 5.5, with and without detergent NP-40 substitute (Fig. 5, Aliquots A2 and B). In addition, the precipitation was also performed in 50 mM NaOH solution adjusted to pH 5.5 with H_3PO_4 instead of phosphate buffer to mimic the original conditions under which full precipitation was reported [26] (Fig. 5, Aliquots C and D).

Both pellet and acetone fractions were collected, analyzed and the recovered amounts were calculated against a calibration curve of Man-5 and Man-9 standards. The different recovery of both glycans was clearly apparent in these experiments, with Man-5 predominantly remaining in the acetone fractions and Man-9 recovered from the pellet fractions. These results thus confirmed the observations made in the glycoprofile precipitation experiments. Of the total amount of 100 ng added, only around 20–30% of the total Man-5 was recovered in the pellet fractions, while the rest remained in the (normally discarded) acetone fractions (Table 3). Recovery of the Man-9 glycan was much higher, with 80–90% recovered from the pellet fractions. The recovery appeared independent of the presence of denaturing agents and the detergent NP-40. Interestingly, recovery was not improved when reproducing the protocol from Verostek *et al.* (2000) (Table 4). Under all conditions tested, the co-precipitated proteins did not influence the recovery. The calculated total amount Man-5 and Man-9 recovered was 75% and 85%, respectively (Table 5). The small remaining amount (15–25%) is likely lost during subsequent processing steps, which include graphitized

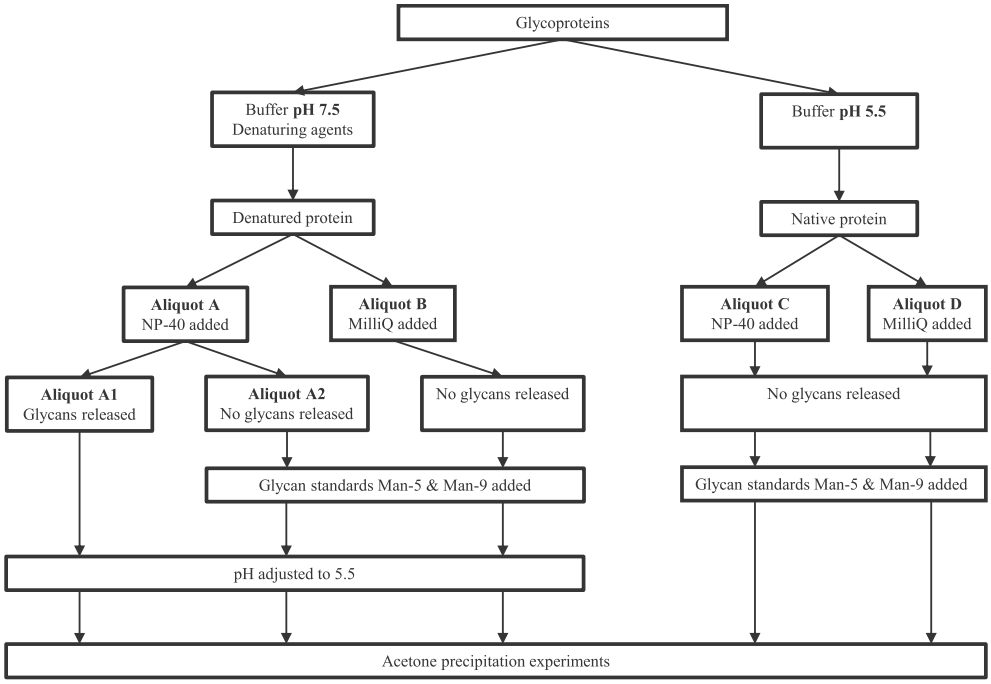


Fig. 5. Schematic overview of the acetone precipitation evaluation experiments. All aliquot treatments and analyses were performed in duplicate.

carbon, labeling and cellulose purification prior to the final analysis.

3. Discussion

For a detailed analysis of N-glycan profiles, samples are commonly purified in order to remove interfering components or to improve sensitivity of analysis. For analytical profiling of a glycoprotein a small amount of glycan (nmol to pmol scale) is sufficient. Efficient removal of interfering lipids, protein and peptides prior to the labeling procedure is usually accomplished in a few or even a single step [21,25]. Trace amounts of lipids and peptides do not interfere with analyses, and residual detergents and salts from the glycan release procedure are usually not a problem either.

Aiming to obtain sufficient amounts of material for functional studies, we developed a protocol for N-glycan isolation on a larger scale. To achieve this, available protocols had to be optimized. In view of the larger volumes used, standard analytical work-up methods were no longer convenient. Together with the target glycans of interest, the amounts of protein and detergent contaminants also increased. The increased solvent volumes, from mL scale to several 100 mL, complicated sample handling. Standard SPE cartridges have a limited capacity for target analytes and contaminants and typically accommodate volumes up to 10 mL. For functional studies all non-glycan components needed to be removed, to avoid non-glycan specific responses. In order to be able to directly relate the observed effects to the presence of the N-glycans, the obtained glycans have to be free of any residual and potentially interfering protein remnants, or residues of the detergents used in the N-glycan release protocol.

In this work, two approaches for removing detergent from a PNGase F glycoprotein digest were compared. Acetone precipitation resulted in

Table 4
Recovery of Man-5 and Man-9 in the pellet and acetone fractions during acetone precipitation in 50 mM NaOH solution (see also Fig. 5). No NP-40 was added to these experiments. Recovery in ng of a total addition of 100 ng of Man-5 and Man-9. Average of duplicate experiments.

	Pellet fraction		Acetone fraction	
	Man-5	Man-9	Man-5	Man-9
RNase B	17 ± 2	69 ± 1	57 ± 9	8 ± 1
Bovine lactoferrin	11 ± 1	66 ± 1	61 ± 3	12 ± 3
Human lactoferrin	14 ± 0	68 ± 4	53 ± 2	8 ± 2
Ovalbumin	12 ± 1	75 ± 0	66 ± 2	8 ± 0
Fetuin	11 ± 3	67 ± 5	61 ± 2	12 ± 2
Thyroglobulin	7 ± 1	60 ± 5	61 ± 3	20 ± 3
α-acid glycoprotein	16 ± 2	66 ± 6	55 ± 0	14 ± 0

Table 3
Recovery of Man-5 and Man-9 in the pellet and acetone fractions during acetone precipitation in phosphate buffer, in the presence or absence of NP-40 (Aliquots A2 and B, Fig. 5). Recovery in ng of a total addition of 100 ng of Man-5 and Man-9. Average of duplicate experiments.

	Pellet fraction				Acetone fraction			
	No NP-40		NP-40 added		No NP-40		NP-40 added	
	Man-5	Man-9	Man-5	Man-9	Man-5	Man-9	Man-5	Man-9
RNase B	23 ± 2	74 ± 2	24 ± 1	75 ± 3	52 ± 1	6 ± 1	47 ± 4	7 ± 0
Bovine lactoferrin	19 ± 1	79 ± 2	22 ± 2	82 ± 3	56 ± 3	9 ± 2	52 ± 4	6 ± 0
Human lactoferrin	20 ± 4	78 ± 1	26 ± 0	85 ± 1	55 ± 4	7 ± 1	51 ± 9	5 ± 1
Ovalbumin	22 ± 1	82 ± 4	24 ± 1	81 ± 1	55 ± 6	7 ± 0	52 ± 5	5 ± 0
Fetuin	26 ± 8	78 ± 3	25 ± 1	80 ± 1	53 ± 6	6 ± 3	53 ± 1	6 ± 1
Thyroglobulin	23 ± 3	81 ± 1	25 ± 2	83 ± 0	53 ± 2	8 ± 2	49 ± 0	6 ± 1
α-acid glycoprotein	22 ± 0	80 ± 1	26 ± 4	80 ± 1	54 ± 2	7 ± 0	50 ± 5	5 ± 0

Table 5
Combined recovery of Man-5 and Man-9 in pellet and acetone fractions. Recovery in ng of a total addition of 100 ng of Man-5 and Man-9. Average of duplicate experiments.

	Phosphate buffer pH 5.5				50 mM NaOH pH 5.5	
	No NP-40		NP-40 added		No NP-40	
	Man-5	Man-9	Man-5	Man-9	Man-5	Man-9
RNase B	75 ± 3	80 ± 3	71 ± 4	82 ± 2	74 ± 11	77 ± 2
Bovine lactoferrin	75 ± 2	88 ± 0	74 ± 2	88 ± 3	72 ± 4	79 ± 2
Human lactoferrin	75 ± 0	85 ± 2	78 ± 9	90 ± 1	68 ± 2	77 ± 6
Ovalbumin	77 ± 6	88 ± 3	75 ± 4	86 ± 1	79 ± 3	84 ± 0
Fetuin	79 ± 2	85 ± 0	78 ± 2	86 ± 0	71 ± 5	79 ± 3
Thyroglobulin	76 ± 1	89 ± 1	75 ± 2	89 ± 0	68 ± 5	79 ± 6
α-acid glycoprotein	76 ± 1	87 ± 1	76 ± 1	85 ± 1	71 ± 2	80 ± 8

a lower overall N-glycan yield when compared to the Bio-Beads SM-2 detergent removal. Furthermore, using acetone precipitation, smaller N-glycans (oligomannose type glycans in particular) remained dissolved in the acetone and were discarded in the normal procedure. This phenomenon was most noticeable for the oligomannose type glycans, but a similar loss was also observed for biantennary sialylated structures (FA2G2S1 and FA2G2S2). Glycans of higher complexity (tri and tetraantennary structures) were recovered fully. These observations are in contradiction with Verostek *et al.* (2000), claiming full recovery of all glycans from the pellet. Recovery in this earlier publication was determined with the phenol-sulfuric acid method. In this method, oligosaccharides are broken down into monosaccharides and converted to furfurals, after which they can react with phenol to form a yellow colored compound [42]. Ketones, such as acetone, will also react with phenol under these circumstances, forming bisphenol A and other side products [43]. While Verostek *et al.* (2000) took care to remove acetone before analysis, a residual yellow color was consistently observed. This may have led to an overestimation of the final carbohydrate recovery. While the radioactive Man5GlcNAc[3 H]-ol was fully recovered in all experiments by Verostek and coworkers, in our work we consistently observed significant losses of $Man_5GlcNAc_2$. Acetone precipitation has been applied with and without addition of NP-40 present in the solvents for glycoprofiling experiments [44,45]. We investigated the effect of added detergent on the precipitation efficacy and found that recovery of $Man_5GlcNAc_2$ from the pellet was < 30% under all conditions tested regardless of added detergent. These results clearly show that caution is needed when applying acetone precipitation for future glycoprofiling experiments. This caution is not limited to the glycans from bovine lactoferrin, but also required for other glycoproteins; underestimations may occur, as seen with the fucosylated and sialylated glycans FA2G2S1 and FA2G2S2. The latter glycans are commonly expressed on human immunoglobulin G (IgG) and these structures are implicated in the functionality of the IgG molecule [46]. Glycoprofile analysis of these IgG glycans is therefore often done by calculating the relative abundances of these glycans in the profile. Due to the uneven precipitation, a profile obtained from an acetone precipitated sample can therefore lead to alternative conclusions when compared to other methods of purification.

Since N-glycan precipitation was proven incomplete, particularly for the smaller oligomannose glycans, this protocol was not ideal for the recovery of glycans from our target protein, bovine lactoferrin. Alternative detergent removal options such as dialysis would lead to loss of the released glycans and ion exchange was not compatible with the non-ionic detergent NP-40. Instead, a hydrophobic interaction sorbent (Bio-Beads SM-2, Bio-Rad) was applied for the removal of NP-40. Upon addition of the Bio-Beads SM-2, NP-40 and SDS were adsorbed onto the sorbent, as reported before [27,47,48].

Extracts of both protocols (acetone precipitation and Bio-Beads SM-2), were further purified by a sequence of C18 and graphitized carbon steps. While glycans are not captured on C18 material, residual protein and peptides are trapped and will not compete with the glycans on the graphitized carbon material. Proteins and peptides have a tendency to bind so strongly to graphitized carbon that they cannot be effectively eluted and only bleed from the material [25]. Addition of the C18 column step therefore also allowed repeated use of the graphitized carbon material, without the risk of saturation and bleed of protein in subsequent uses.

Residual detergent was still detected after C18 and graphitized carbon SPE steps but was removed by a final wash of lyophilized glycans with 100% acetone. The final purity obtained with both protocols described in this work was 89–100% based on monosaccharide analysis and no protein and detergent traces were detected with 1D ^{1}H NMR spectroscopy analysis. This makes the obtained N-glycans suitable for functional analysis studies.

When choosing between the two methods of cleanup, a careful evaluation has to be made based on the types of glycans present on the glycoprotein of interest. As demonstrated in this work, acetone precipitation is most suited for larger complex-type glycans. Smaller glycans, especially of the oligomannose variety, are partially precipitated, in a non-homogeneous manner. An example of a biologically relevant protein decorated with oligomannose glycans is lactoferrin. Previously we have shown that modifications to the profile of the oligomannose glycans of lactoferrin alter the functionality of this glycoprotein [9]. To correctly analyze the functionality of glycans from different lactoferrin sources, preserving the complete glycoprofile is important. In this case, the Bio-Beads SM-2 detergent removal was proven to be more suitable. The Bio-Beads SM-2 protocol is also suited for samples with multiple proteins, such as a whey protein digest or for proteins with an unknown glycosylation profile. As no glycans are selectively discarded, this protocol ensures that the full profile is conserved.

Methods for the isolation of N-glycans in a high yield and purity have been described before. However, these are limited to either purification of labeled glycans [23], or are released by Endo-N-acetylglucosaminidase enzymes, such as Endo-B1 [17]. While glycans released by Endo-B1 are more easily purified, this approach has some limitations. The reaction conditions used during the glycan release by endo-B1 influence the glycan types that are released (Parc *et al.*, 2015). When studying the biological effects of the complete glycoprofile of a particular protein, a full release of all glycans in a single reaction is preferred. With the protocol described in this work, full glycoprofiles of native glycans from any glycoprotein can be purified and used for subsequent functional analysis.

4. Materials and methods

4.1. Materials

RNase B (Bovine pancreas), Ovalbumin, fetuin (fetal calf serum), thyroglobulin (porcine) and human lactoferrin were from Sigma-Aldrich Chemie N.V. (Zwijndrecht, the Netherlands). Human alpha-1-

acid glycoprotein (AGP) was a gift from J. P. Kamerling (Utrecht University) and was originally obtained from Dade Behring (Marburg, Germany). Bovine lactoferrin was provided by FrieslandCampina Domo (Amersfoort, the Netherlands). PNGase F (*Flavobacterium meningosepticum*) was from New England Biolabs (Ipswich, UK). Bio-Beads SM-2 were purchased from Bio-Rad Laboratories (Veenendaal, the Netherlands). Maltohexaose and maltoheptaose of ≥90% purity were from Sigma-Aldrich. N-linked glycan standards $Man_5GlcNAc_2$ (Man-5) and $Man_9GlcNAc_2$ (Man-9) were from Ludger Ltd. (Oxfordshire, UK). Acetone (ACS reagent grade) was from Sigma-Aldrich.

Solid phase extraction (SPE) of the large-scale PNGase F digests was performed on sequentially connected individual columns with 5 g of C18 (SiliaBond C18 WPD, 37–55 μm, 125 Å, SiliCycle) and 5 g of graphitized carbon material (Carbon Graph, Non-Porous 120/400 Mesh, Screening Devices, Amersfoort, the Netherlands). Alternatively, prepacked C18 (CEC18, 200 mg/3 mL, Screening Devices, Amersfoort, the Netherlands) and graphitized carbon SPE cartridges (Extract-clean carbograph, 150 mg/4 mL, Grace, Columbia, USA) were used.

4.2. Glycan release

Up to 1 g bovine lactoferrin per incubation was dissolved at a concentration of 2.5–7.5 mg/mL in 100 mM sodium phosphate buffer (pH 7.5). SDS was added at a 1:1 w/w protein: SDS ratio and β-mercaptoethanol (Sigma) was added to a concentration of 1% v/v. The protein was denatured by heating at 85 °C for 30 min. Denatured protein was alkylated by addition of iodoacetamide (Sigma) to a concentration of 20 mM (55 °C; 30 min). Nonidet P-40 substitute (NP-40, Sigma) was added at a final concentration of 1% v/v. PNGase F was added at a concentration of 50 U/mg glycoprotein and the solution incubated overnight at 37 °C with continuous agitation. Completion of the digestion was confirmed by SDS-PAGE using 0.5 mm thick 10% acrylamide gels, stained with Bio-Safe Coomassie G-250 (Bio-Rad).

4.3. Detergent and protein removal

4.3.1. Large-scale acetone precipitation (approach A)

Large-scale PNGase F digests of up to a gram of protein were acidified to pH 5.5 using 2 M HCl and divided into 10 mL aliquots in 50 mL polypropylene tubes. A volume of 40 mL of ice-cold (−20 °C) acetone was added (final acetone concentration 80%) and the samples homogenized. The tubes were stored at −20 °C for at least 1 h followed by centrifugation for 1 h at 4000 × *g* and 4 °C. The acetone fraction was carefully removed. The pellet was first triturated with a minimal amount of ice cold 60% methanol, and then suspended with another 5 mL ice cold 60% methanol. The mixture was stored at −20 °C overnight and centrifuged (4 °C, 4000 × *g*, 1 h). The 60% methanol fractions were collected and the methanol evaporated under N_2. The extracts were stored at −20 °C until further cleanup by C18 and graphitized carbon SPE steps.

4.3.2. Acetone precipitation evaluation experiments, (small scale)

Precipitation experiments were performed with the glycoproteins RNase B, ovalbumin, fetuin, thyroglobulin, human lactoferrin, bovine lactoferrin. Proteins either were dissolved at a concentration of 1 mg/mL in 100 mM phosphate buffer at pH 7.5 and denatured and alkylated as described earlier, or directly dissolved in 50 mM phosphate buffer at pH 5.5. Protein solutions were divided into amounts of 200 μg of protein, in duplicate per aliquot (Fig. 5). NP-40 was either added at a concentration of 1% (Aliquot A) or substituted by MilliQ (Aliquot B). PNGase F (50 U/mg) was only added to Aliquot A1 (Fig. 5) resulting in release of glycans from denatured proteins (with added NP-40). To all other aliquots (A2, B, C, D; Fig. 5), 100 ng amounts of the Man-5 and Man-9 glycan standards were added. Acetone precipitation of the proteins and glycans was performed according to the procedure of Verostek *et al.* In short, after adjusting the pH to 5.5 with 10% H_3PO_4 and addition of ice-cold acetone, digests were stored at −20 °C overnight. Centrifugation was performed at 13000 × *g* for 20 min at +4 °C. The acetone fraction was carefully collected, evaporated under N_2 and redissolved in MilliQ water. The complete pellet was triturated and suspended in 1 mL of MilliQ water. Glycans were isolated from the acetone and pellet fractions by graphitized carbon SPE. All experiments were performed in duplicate.

4.3.3. Bio-Beads SM-2 protocol (approach B)

Bio-Beads SM-2 were added to PNGase F protein digests at a ratio of 1 g of beads: 10 mg of digested protein to remove detergents. Samples were stirred for 3 h at room temperature to allow adsorption of NP-40 and SDS onto the beads. Supernatant with N-glycans was collected and an aliquot of MilliQ was added in a 1 : 1 ratio to the used beads. This MilliQ fraction was collected and combined with the first supernatant fraction. Soluble protein in the combined fractions was removed by filtration over 30 kDa centrifugal MWCO filters (Amicon Ultra, Merck Millipore, Tullagreen, Cork, IRL).

The Bio-Beads SM-2 were re-used for duplicate incubations of the same protein after a cleaning cycle (Supplemental Information).

4.4. Solid phase extraction

Graphitized carbon solid phase extraction (SPE) of the acetone precipitation evaluation experiments was performed according to the procedure of Packer *et al.* [25]. Elution fractions were neutralized with 2% ammonia and acetonitrile was evaporated using a Speedvac Savant 131DDA sample concentrator (Thermo Fisher Scientific, Waltham, MA) followed by lyophilization.

Large scale digests from Approach A or Approach B were further purified by a sequence of C18 and graphitized carbon SPE to remove residual protein and salts. Digests partially purified by Approach A were further processed on sequentially connected individual columns with 5 g of C18 (SiliaBond C18 WPD, 37–55 μm, 125 Å, SiliCycle) and 5 g of graphitized carbon material (Carbon Graph, Non-Porous 120/400 Mesh, Screening Devices, Amersfoort, the Netherlands). Alternatively, for digests from Approach B, prepacked C18 (CEC18, 200 mg/3 mL, Screening Devices, Amersfoort, the Netherlands) and graphitized carbon SPE cartridges (Extract-clean carbograph, 150 mg/4 mL, Grace, Columbia, USA) were used, with the digest split into a 50 mg (partially purified) digest aliquots.

The full procedure, including material conditioning and wash steps, is described in the Supplemental Information. In short, aqueous glycan samples were loaded onto conditioned C18 material and the flow through, containing glycans, was collected and loaded onto the graphitized carbon. The graphitized carbon was washed with MilliQ water to remove salts and finally the glycans were eluted with 25% acetonitrile containing 0.1% TFA. Elution fractions were neutralized with 2% ammonia, the acetonitrile evaporated under N_2 and lyophilized.

4.5. Final purification of isolated N-glycans (acetone wash)

Lyophilized N-glycans were washed with 5 mL 100% ice cold (−20 °C) acetone to remove trace detergents. The pellets were disturbed by the addition of a small magnetic stirrer and stirred vigorously for 10 min until they were dispersed into fine powdered suspensions. After centrifugation (4000 × *g*, 5 min, 4 °C), the acetone was carefully removed and the process repeated for a total of 5 times. The washed pellets were dissolved in a small quantity of MilliQ, transferred to preweighed tubes and lyophilized. Weight of the purified glycans was determined by re-weighing the tube after lyophilization. Purity of the resulting glycan products were determined by monosaccharide analysis and 1D ^1H NMR spectroscopy.

4.6. Glycan labeling (2-AA)

Isolated glycans were labeled with anthranilic acid (2-AA, Sigma). Lyophilized glycan samples were dissolved in labeling solution at a minimum ratio of 10 μL of labeling solution to 10 μg of glycan. Labeling solution consisted of 0.35 M of 2-AA and 1 M sodium cyanoborohydride (Sigma) in dimethylsulfoxide (DMSO, Sigma): glacial acetic acid (7:3, v/v) and incubations were performed for 2 h at 65 °C [49]. Labeling reagents were removed by 96-well microcrystalline cellulose SPE as described [21].

4.7. HPLC analysis

2-AA labeled glycans were separated on an Acquity UPLC Glycan BEH Amide column (2.1 mm × 100 mm, 1.7 μm, Waters, Etten-Leur, the Netherlands), using a UltiMate 3000 SD HPLC system (Thermo Fisher Scientific, Waltham, MA) equipped with a Jasco FP-920 fluorescence detector (λex 330 nm, λem 420 nm, Jasco Inc, Easton, MD). An injection volume of 1 μL was used for protein glycoprofiles and 3 μL for the Man-5 & Man-9 recovery experiments. For quantification, a 5-point calibration curve of 2-AA labeled Man-5 and Man-9 ranging from 25 to 500 ng was used.

Ternary gradients were run using MilliQ, acetonitrile and 250 mM formic acid adjusted to pH 3.0 using ammonia. A MilliQ gradient was used from of 25%–35% MilliQ (total concentration) for 45 min, or 25%–40% MilliQ for 67.5 min at a flow rate of 0.5 mL/min, maintaining an identical slope in both gradients. A constant 20% of the formic acid solution at pH 3.0 was maintained throughout the run. The remaining percentage of the solvent composition comprised of acetonitrile. Selection between the 45 or 67.5 min gradient was made based on the complexity of the glycan profile. Bovine and human lactoferrin, RNase B, ovalbumin glycoprofiles were analyzed with the 45 min gradient. Profiles of fetuin, α-acid glycoprotein and thyroglobulin were analyzed using the longer 67.5 min gradient. After completion of the gradient, final gradient conditions were maintained for 9 min and the column reconditioned back to initial conditions for 13 min.

4.8. Monosaccharide analysis

Aliquots of 0.1 mg of purified glycan sample were subjected to methanolysis in 1.0 M methanolic HCl (Sigma) for 24 h at 85 °C. The resulting monosaccharides were re-*N*-acetylated and trimethylsilylated. Analysis of the trimethylsilylated (methyl ester) methyl glycosides was performed by GLC on a Restek RTX-1 column (30 m × 0.25 mm; Restek Corporation, Bellefonte, PA), using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA; temperature program 140–225 °C, 6 °C/min). Confirmation of the monosaccharide identities was performed by GC-MS analysis on a Shimadzu QP2010 Plus system (Shimadzu, 's Hertogenbosch, The Netherlands), using an ZB-1HT column (30 m × 0.25 mm, Phenomenex, Torrance, CA; temperature program 140–240 °C, 8 °C/min) [50].

4.9. One-dimensional ^1H NMR spectroscopy

Purified glycan samples (~1 mg) were lyophilized and exchanged twice with 99.9% D_2O (Cambridge Isotope Laboratories Inc., Andover, MA) and subsequently dissolved in 650 μL of D_2O, containing acetone as internal standard (δ^1H 2.225). Resolution-enhanced one-dimensional 500 MHz ^1H NMR spectra were recorded in D_2O on a Varian Inova 500 spectrometer (GBB, NMR Center, University of Groningen) at probe temperatures of 25 °C, with a spectral width of 4000 Hz, collecting 16k complex data points. A WET1D pulse was applied to suppress the HOD signal. Spectra were processed with MestReNova 12 (Mestrelabs Research SL, Santiago de Compostella, Spain).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2019.04.011.

References

[1] K.W. Moremen, M. Tiemeyer, A.V. Nairn, Vertebrate protein glycosylation: diversity, synthesis and function, Nat. Rev. Mol. Cell Biol. 13 (2012) 448–462, https://doi.org/10.1038/nrm3383.
[2] A. Varki, Biological roles of glycans, Glycobiology 27 (2017) 3–49, https://doi.org/10.1093/glycob/cww086.
[3] E. Varki A, R.D. Cummings, J.D. Esko, et al., Essentials of Glycobiology, third ed., (2015).
[4] V. Coelho, S. Krysov, A.M. Ghaemmaghami, M. Emara, K.N. Potter, P. Johnson, G. Packham, L. Martinez-Pomares, F.K. Stevenson, Glycosylation of surface Ig creates a functional bridge between human follicular lymphoma and microenvironmental lectins, Proc. Natl. Acad. Sci. 107 (2010) 18587–18592, https://doi.org/10.1073/pnas.1009388107.
[5] Y.G. Kim, D.S. Shin, Y.H. Yang, G.C. Gil, C.G. Park, Y. Mimura, D.K.C. Cooper, P.M. Rudd, R.A. Dwek, Y.S. Lee, B.G. Kim, High-Throughput screening of glycan-binding proteins using miniature pig kidney N-Glycan-Immobilized beads, Chem. Biol. 15 (2008) 215–223, https://doi.org/10.1016/j.chembiol.2008.02.009.
[6] D. Chui, G. Sellakumar, R. Green, M. Sutton-Smith, T. McQuistan, K. Marek, H. Morris, A. Dell, J. Marth, Genetic remodeling of protein glycosylation in vivo induces autoimmune disease, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 1142–1147, https://doi.org/10.1073/pnas.98.3.1142.
[7] J.C.M. De Freitas, B.D.R.D.A. Silva, W.F. De Souza, W.M. De Araújo, E.S.F.W. Abdelhay, J.A. Morgado-Díaz, Inhibition of N-linked glycosylation by tunicamycin induces E-cadherin-mediated cell-cell adhesion and inhibits cell proliferation in undifferentiated human colon cancer cells, Canc. Chemother. Pharmacol. 68 (2011) 227–238, https://doi.org/10.1007/s00280-010-1477-8.
[8] L. Dissing-Olesen, M. Thaysen-Andersen, M. Meldgaard, P. Højrup, The function of the human interferon-Beta1a glycan determined in vivo, J. Pharmacol. Exp. Ther. 326 (2008) 338–347, https://doi.org/10.1124/jpet.108.138263.IFN.
[9] S. Figueroa-Lozano, R.L. Valk-Weeber, S.S. van Leeuwen, L. Dijkhuizen, P. de Vos, Dietary N-glycans from bovine lactoferrin and TLR modulation, Mol. Nutr. Food Res. 62 (2018) 1–30, https://doi.org/10.1002/mnfr.201700389.
[10] R.M. Anthony, F. Nimmerjahn, D.J. Ashline, V.N. Reinhold, J.C. Paulson, J. V Ravetch, Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc, Science 320 (2008) 373–376, https://doi.org/10.1126/science.1154315.
[11] Man Sung Co, D.A. Scheinberg, N.M. Avdalovic, K. Mcgraw, M. Vasquez, P.C. Caron, C. Queen, Genetically engineered deglycosylation of the variable domain increases the affinity of an anti-CD33 monoclonal antibody, Mol. Immunol. 30 (1993) 1361–1367, https://doi.org/10.1016/0161-5890(93)90097-U.
[12] L.F. Iversen, J.S. Kastrup, S.E. Bjørn, F.C. Wiberg, I.K. Larsen, H.J. Flodgaard, P.B. Rasmussen, Structure and function of the N-linked glycans of HBP/CAP37/azurocidin: crystal structure determination and biological characterization of nonglycosylated HBP, Protein Sci. 8 (1999) 2019–2026, https://doi.org/10.1110/ps.8.10.2019.
[13] A.S.B. Edge, Deglycosylation of glycoproteins with trifluoromethanesulphonic acid: elucidation of molecular structure and function, Biochem. J. 376 (2003) 339–350, https://doi.org/10.1042/bj20030673.
[14] L. Bode, Human milk oligosaccharides: every baby needs a sugar mama, Glycobiology 22 (2012) 1147–1162, https://doi.org/10.1093/glycob/cws074.
[15] G.T. Macfarlane, H. Steed, S. Macfarlane, Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics, J. Appl. Microbiol. 104 (2008) 305–344, https://doi.org/10.1111/j.1365-2672.2007.03520.x.
[16] F. Capitan-Canadas, M. Ortega-Gonzalez, E. Guadix, A. Zarzuelo, M.D. Suarez, F.S. de Medina, O. Martinez-Augustin, Prebiotic oligosaccharides directly modulate proinflammatory cytokine production in monocytes via activation of TLR4, Mol. Nutr. Food Res. 58 (2014) 1098–1110, https://doi.org/10.1002/mnfr.201300497.
[17] S. Karav, A. Le Parc, J.M. Leite Nobrega de Moura Bell, S.A. Frese, N. Kirmiz, D.E. Block, D. Barile, D.A. Mills, Oligosaccharides released from milk glycoproteins are selective growth substrates for infant-associated bifidobacteria, Appl. Environ. Microbiol. 82 (2016) 3622–3630, https://doi.org/10.1128/AEM.00547-16.
[18] J.N. Arnold, M.R. Wormald, R.B. Sim, P.M. Rudd, R.A. Dwek, The impact of glycosylation on the biological function and structure of human immunoglobulins, Annu. Rev. Immunol. 25 (2007) 21–50, https://doi.org/10.1146/annurev.immunol.25.022106.141702.
[19] F. Clerc, K.R. Reiding, B.C. Jansen, G.S.M. Kammeijer, A. Bondt, M. Wuhrer, Human plasma protein N-glycosylation, Glycoconj. J. 33 (2016) 309–343, https://doi.org/10.1007/s10719-015-9626-2.
[20] T. Patel, J. Bruce, a Merry, C. Bigge, M. Wormald, a Jaques, R. Parekh, Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins, Biochemistry 32 (1993) 679–693, https://doi.org/

10.1021/bi00053a037.
[21] L.R. Ruhaak, C. Huhn, W.J. Waterreus, A.R. De Boer, C. Neusüss, C.H. Hokke, A.M. Deelder, M. Wuhrer, Hydrophilic interaction chromatography-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins, Anal. Chem. 80 (2008) 6119–6126, https://doi.org/10.1021/ac800630x.
[22] M.H.J. Selman, M. Hemayatkar, A.M. Deelder, M. Wuhrer, Cotton HILIC SPE microtips for microscale purification and enrichment of glycans and glycopeptides, Anal. Chem. 83 (2011) 2492–2499, https://doi.org/10.1021/ac1027116.
[23] W.R. Alley, B.F. Mann, V. Hruska, M.V. Novotny, Isolation and purification of glycoconjugates from complex biological sources by recycling high-performance liquid chromatography, Anal. Chem. 85 (2013) 10408–10416, https://doi.org/10.1021/ac4023814.
[24] I. Burnina, E. Hoyt, H. Lynaugh, H. Li, B. Gong, A cost-effective plate-based sample preparation for antibody N-glycan analysis, J. Chromatogr. A. 1307 (2013) 201–206, https://doi.org/10.1016/j.chroma.2013.07.104.
[25] N.H. Packer, M. a. Lawson, D.R. Jardine, J.W. Redmond, A general approach to desalting oligosaccharides released from glycoproteins, Glycoconj. J. 15 (1998) 737–747, https://doi.org/10.1023/A:1006983125913.
[26] M.F. Verostek, C. Lubowski, R.B. Trimble, Selective organic precipitation/extraction of released N-glycans following large-scale enzymatic deglycosylation of glycoproteins, Anal. Biochem. 278 (2000) 111–122, https://doi.org/10.1006/abio.1999.4433.
[27] J.-L. Rigaud, D. Lévy, M.G, O. Lambert, Detergent removal by Bio-Beads. Application to membrane protein reconstitution and two-dimensional crystallisation, Eur. J. Biophys. 27 (1998) 305–319.
[28] P.S.J. Cheetham, Removal of Triton X-100 from aqueous solution using amberlite XAD-2, Anal. Biochem. 92 (1979) 447–452, https://doi.org/10.1016/0003-2697(79)90683-3.
[29] S.S. Van Leeuwen, R.J.W. Schoemaker, C.J. a M. Timmer, J.P. Kamerling, L. Dijkhuizen, Use of Wisteria floribunda agglutinin affinity chromatography in the structural analysis of the bovine lactoferrin N-linked glycosylation, Biochim. Biophys. Acta Gen. Subj. 1820 (2012) 1444–1455, https://doi.org/10.1016/j.bbagen.2011.12.014.
[30] B. Coddevilie, G. Strecker, J. Wieruszeski, Heterogeneity of bovine lactotransferrin glycans, Carbohydr. Res. 236 (1992) 145–164.
[31] B. Donnelly, Reactions of oligosaccharides. 3. Hygroscopic properties, Cereal Chem. 50 (1973) 512–519.
[32] D. Fu, L. Chen, R.A. O'Neill, A detailed structural characterization of ribonuclease B oligosaccharides by1H NMR spectroscopy and mass spectrometry, Carbohydr. Res. 261 (1994) 173–186, https://doi.org/10.1016/0008-6215(94)84015-6.
[33] N. Kawasaki, M. Ohta, S. Hyuga, O. Hashimoto, T. Hayakawa, Analysis of carbohydrate heterogeneity in a glycoprotein using liquid chromatography/mass spectrometry and liquid chromatography with tandem mass spectrometry, Anal. Biochem. 269 (1999) 297–303, https://doi.org/10.1006/abio.1999.4026.
[34] D.J. Harvey, D.R. Wing, B. Küster, I.B.H. Wilson, Composition of N-linked carbohydrates from ovalbumin and co-purified glycoproteins, J. Am. Soc. Mass Spectrom. 11 (2000) 564–571, https://doi.org/10.1016/S1044-0305(00)00122-7.
[35] T. Tsuji, K. Yamamoto, T. Irimura, T. Osawa, Structure of carbohydrate unit A or porcine thyroglobulin, Biochem. J. 195 (1981) 691–699 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1162942/.
[36] K. Yamamoto, T. Tsuji, T. Irimura, T. Osawa, The structure of carbohydrate unit B of porcine thyroglobulin, Biochem. J. 195 (1981) 701–713, https://doi.org/10.1042/bj1950701.
[37] G. Spik, G. Strecker, B. Fournet, S. Bouquelet, J. Montreuil, L. Dorland, H. van Halbeek, J.F.G. Vliegenthart, Primary structure of the glycans from human lactotransferrin, Eur. J. Biochem. 121 (1982) 413–419, https://doi.org/10.1111/j.1432-1033.1982.tb05803.x.
[38] E. Balaguer, C. Neusüss, Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis-electrospray ionization-mass spectrometry, Anal. Chem. 78 (2006) 5384–5393, https://doi.org/10.1021/ac060376g.
[39] X. Sun, L. Tao, L. Yi, Y. Ouyang, N. Xu, D. Li, R.J. Linhardt, Z. Zhang, N-glycans released from glycoproteins using a commercial kit and comprehensively analyzed with a hypothetical database, J. Pharm. Anal. 7 (2017) 87–94, https://doi.org/10.1016/j.jpha.2017.01.004.
[40] M. Melmer, T. Stangler, A. Premstaller, W. Lindner, Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis, J. Chromatogr. A. 1218 (2011) 118–123, https://doi.org/10.1016/j.chroma.2010.10.122.
[41] M.J. Treuheit, C.E. Costello, H.B. Halsall, Analysis of the five glycosylation sites of human alpha 1-acid glycoprotein, Biochem. J. 283 (Pt 1) (1992) 105–112, https://doi.org/10.1002/jms.938.
[42] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356, https://doi.org/10.1021/ac60111a017.
[43] F.W. Neumann, W.E. Smith, By-product of the bisphenol a reaction. Syntheses and structure assignments of 2,4'-isopropylidenediphenol and 4,4'-(4-Hydroxy-m-phenylenediisopropylidene)- diphenol, J. Org. Chem. 31 (1966) 4318–4320, https://doi.org/10.1021/jo01350a552.
[44] S. Kim, S.K. Hwang, R.A. Dwek, P.M. Rudd, Y.H. Ahn, E.H. Kim, C. Cheong, S. Il Kim, N.S. Park, S.M. Lee, Structural determination of the N-glycans of a lepidopteran arylphorin reveals the presence of a monoglucosylated oligosaccharide in the storage protein, Glycobiology 13 (2003) 147–157, https://doi.org/10.1093/glycob/cwg023.
[45] C.E. Costello, J.M. Contado-Miller, J.F. Cipollo, A glycomics platform for the analysis of permethylated oligosaccharide alditols, J. Am. Soc. Mass Spectrom. 18 (2007) 1799–1812, https://doi.org/10.1016/j.jasms.2007.07.016.
[46] C. Raymond, A. Robotham, M. Spearman, M. Butler, J. Kelly, Y. Durocher, Production of alpha2,6-sialylated IgG1 in CHO cells, mAbs 7 (2015) 571–583, https://doi.org/10.1080/19420862.2015.1029215.
[47] J.L. Fox, S.E. Stevens, C.P. Taylor, L.L. Poulsen, SDS removal from protein by polystyrene beads, Anal. Biochem. 87 (1978) 253–256, https://doi.org/10.1016/0003-2697(78)90591-2.
[48] T. Momoi, The presence of lipophilic glycoprotein interacting with insulin, Biochem. Biophys. Res. Commun. 87 (1979) 541–549, https://doi.org/10.1016/0006-291X(79)91829-1.
[49] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Nonselective and efficient fluorescent labeling of glycans using 2-amino Benzamide and anthranilic acid, Anal. Biochem. 230 (1995) 229–238 https://doi.org/10.1006/abio.1995.1468.
[50] J.P. Kamerling, J.F.G. Vliegenthart, Carbohydrates, in: A.M. Lawson (Ed.), Clin. Biochem. – Princ. Methods, Appl. Mass Spectrom, vol. 1, Walter de Gruyter, Berlin, 1989, pp. 176–263.
[51] J. Ahn, J. Bones, Y.Q. Yu, P.M. Rudd, M. Gilar, Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 403–408, https://doi.org/10.1016/j.jchromb.2009.12.013.
[52] J. Sjögren, W.B. Struwe, E.F.J. Cosgrave, P.M. Rudd, M. Stervander, M. Allhorn, A. Hollands, V. Nizet, M. Collin, EndoS $_2$ is a unique and conserved enzyme of serotype M49 group A *Streptococcus* that hydrolyses N-linked glycans on IgG and α_1 -acid glycoprotein, Biochem. J. 455 (2013) 107–118, https://doi.org/10.1042/BJ20130126.